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Phenotypic, Genotypic and DNA Sequencing Detection of Pathogenic Molds Isolated from Different Clinical Cases of Immunocompromised Patients

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ABSTRACT

Background: *Pathogenic fungi are infectious agents of great concern for immunocompromised persons. Timely and accurate identification of pathogenic molds is critical for their effective detection and treatment. This study aimed to characterize the species of mold isolated from clinical samples using phenotypic, genotypic, and molecular methods and compare them with reference sequences from international repositories.* ***Objectives:*** *To create a local repository of mold species infecting immunocompromised patients, undertake genetic characterization, and determine relatedness to standard strains as documented in NCBI.* ***Methods:*** *From February to April 2022, a total of 256 clinical samples (blood, urine, oral swabs, and sputum) were collected from Al-Sadder Medical City in Al-Najaf province. Phenotypic identification was performed using standard morphological techniques on SDA medium. Molecular identification included PCR amplification of the aspergillopepsin gene with PEPO primers and sequencing of some isolates.* ***Results:*** *Mold growth was positive in 88 out of 256 samples with a frequency of 34.4 percent in blood (49), urine (25), sputum (10), and oral swabs (4). A total of five genera of fungi were identified and most of them were Aspergillus spp. at 70.4 percent, followed by Penicillium (20.4%), Cladosporium (5.6%), Alternaria (2.2%), and Curvularia (1.1%). Within Aspergillus isolates, A. flavus was the most prevalent at 69.3 percent. Of the 20 PCR tested Aspergillus isolates, all were identified as A. flavus genetically. A high identity (98–99%) of the DNA sequence with A. flavus strain AFLA-EME-EG-1 obtained from NCBI was confirmed.* ***Conclusion:*** *The authors consider this finding along with A. flavus being dominant in mold infections in clinical settings of immunosuppressed patients as well as the precise identification using molecular tools underscores the need of further study. The developed local databases are relevant for clinical diagnosis as well as global reference strains performed further explore concordance enhancing their accuracy.*

Keywords: *Aspergillus flavus*, Molecular identification, Immunocompromised and PCR.

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INTRODUCTION

Fungal infections pose a serious threat to public health, especially in immune-compromised patients. Their diagnosis and treatment are challenging because of biological similarities between the fungi and the host cells, which makes identifying the pathogen and choosing suitable antifungal treatment very difficult (1). Among the numerous pathogenic fungi, the Aspergillus species are most worrisome due to their wide distribution in the environment and the risk of causing invasive infections in those with immunosuppression or chronic lung disease (2,3). Aspergillus spores are readily available in soil and decaying vegetation and are

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acquired through inhalation which may result in pulmonary or systemic aspergillosis (4). Fungal taxonomy has traditionally depended on morphology and culture. These approaches often fail to resolve closely related species differentiate (5). Molecular methods, particularly polymerase chain reaction (PCR) and DNA sequencing, have become indispensable in accurately diagnosing fungal pathogens. These approaches enable detection of fungal DNA from clinical samples and cross-matched with reference databases, including those kept at National Center for Biotechnology Information (NCBI) (6,7). The process of DNA sequencing, which is the identification of nucleotides in a DNA molecule, is pivotal in basic science and applied medicine (8). However, information on fungal species related to clinical infections in Iraq is scant. This study aims to fill this gap by recovering molds from different clinical cases of immunocompromised patients, identifying the molds using phenotypic and molecular methods, and analyzing their genetic profiles relative to global standard strains.

METHODS

Sample Collection

Between February and April 2022, a total of 256 clinical samples were gathered from patients with weakened immune systems and different clinical conditions at Al-Sadder Medical City located in Al-Najaf province. The samples consisted of blood (n = 137), urine (n = 57), oral swabs (n = 32), and sputum (n = 30). All specimens were immediately taken to the microbiology laboratory for diagnostics and fungal isolation (1,2).

Identification of Fungal Isolates

Fungal isolates were cultivated on Sabouraud Dextrose Agar (SDA) and incubated for 5-7 days at 28°C. The macroscopic features which included the colony morphology, the color, the texture, and pigmentation were noted. Microscopic examination of the lactophenol cotton blue stain preparation was done to evaluate taxonomically significant hyphal structures, spores, and other relevant structures (3).

Molecular Identification

DNA Extraction

Genomic DNA was extracted from young mold colonies using EZ-10 Spin Column Fungal Genomic DNA Mini Preps Kit (Favorgen Biotech Corp., Taiwan) as per the instructions provided by the manufacturer. In summary, fungal biomass was resuspended in FA buffer, lyticase was applied, and enzymatic as well as mechanical digestion was performed. The lysate was then applied to a silica column and successively washed with W1 and wash buffers. DNA was eluted with either the elution buffer or nuclease-free water and stored at –20°C until further processing (4).

Assessing DNA Quantification and Quality

Extracted DNA was quantified and assessed in the BioDrop spectrophotometer. The quality of the DNA was measured using the absorbance ratio A260/A280, which was within the acceptable limits of 1.7 ± 0.2. The mean concentration of DNA was measured to be 74.65 ng/µL (5).

Preparation of Primers

Aspergillus-specific primers for the aspergillopepsin gene (PEPO) were ordered from Bioneer Corporation, Korea. The received dried primers were reconstituted with deionized distilled water to achieve the concentration of 100 pmol/µL stock solution. Working solutions were made from the stock by diluting it to 10 pmol/µL (6).

PCR Amplification

Amplification was done in a 20 µL reaction mixture containing 5 µL master mix, 5 µL of DNA template, 2.5 µL of each forward and reverse primers, and 5 µL of deionized water. Thermal cycling commenced with an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of 30 seconds of denaturation at 95°C, 30 seconds at 57.5°C for annealing, 72°C extension for 25 seconds, and a final extension at 72°C for 5 minutes (6).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze the PCR products. An agarose gel at 1.3% concentration was made in 1X TBE buffer and stained with ethidium bromide. The electrophoresis was performed at 80 volts for 1 hour. The DNA bands were visualized with a UV transilluminator and captured with a digital imaging system (7,13).

DNA Sequencing

Aspergillus spp. PCR products were purified and processed by Macrogen USA. The fragments were Sanger sequenced, determining the nucleotide sequence of the aspergillopepsin gene. BioEdit software was used for sequence alignments and analyses, and the identity confirmation was performed using NCBI BLAST databases (8,9).

Primers selection

All primers in this study were synthesized by Bioneer company, Korea as following in Table 1).

PCR Program

The general PCR program was listed in Table (3). The amplified PEPO PCR products and DNA ladders were verified by agarose gel electrophoresis to check if the target PEPO gene was present. The identification of the gene was done by comparing the location of the DNA bands with the molecular weight markers. For the analysis, agarose gel was prepared by adding 1.3 grams of agarose into 100 mL of 1X TBE buffer, heating it to complete dissolution, then cooling it to about 55 degrees centigrade before adding 5 µL of Ethidium bromide. This solution was poured into gel trays where it was allowed to solidify with combs inserted to create sample wells. Each well was filled with 5 µL of DNA sample and electrophoresis was performed in 1X TBE buffer at 80 volts for one hour.

The DNA bands were visualized with a UV transilluminator and documented digitally (7). After the PEPR PCR of Aspergillus spp, the products were purified using Promega's PCR purification kit, followed by Sanger sequencing at Macrogen Lab in the USA. Sequence information was generated by Sanger sequencing and processed with BioEdit for multiple sequence alignments. Reference sequences from the NCBI database were utilized for confirming species identification through comparison with the sequences obtained (8).

PCR mixture

The optimization of PCR was conducted after multiple efforts with a PCR mixture, as detailed in (Table 2) from Bioneer Company, Korea.

Table (1): Primer used for Aspergillus spp. detecting of aspergillopepsin gene (5).

Primer	DNA sequence (5'-3')
PEPO- F	5-CGA CGT CTA CAA GCC TTC TGG AAA-3'
PEPO- R	5 -CAG CAG ACC GTC ATT GTT CTT GTC-3'

Table (2): The mixture of PCR.

Mixture solution	Volume
Master mixture	5µl
DNA template	5µl
Forward primers	2.5µl
Reverse primers	2.5µl
Deionized water (dd water)	5µl
Final volume 20	µl

Table (3): PCR Thermal Cycling Conditions for Amplification of the PEPO Gene.

Gene Name	Temperature (ºC) / Time					Cycles Number
	Initial	Cycling Conditions			Final	
	Denaturation	Denaturation	Annealing	Extension	Extension	
PEPO	95/3Min	95/30 sec	57.5/30sec	72/25sec	72/5min	30

RESULTS

Sample Collection and Mold Isolation

Immunocompromised patients with diverse infections yielded a total of 256 clinical samples, blood (53.5%), urine (22.2%), oral swabs (12.5%), and sputum (11.7%) **(Table 4).** Among these, 88 samples (34.4%) demonstrated positive mold growth. Sharps showed the highest rates of positive growth, blood (55.6%), urine (28.4%), sputum (11.3%), and oral swabs (4.5%) **(Table 5).**

Distribution of Fungal Genera

Analysis of 88 positive samples detected 5 fungal genera. Most frequently isolated were Aspergillus spp. (70.4%), followed by Penicillium spp. (20.4%), Cladosporium spp. (5.6%), Alternaria spp. (2.2%), and Curvularia spp. (1.1%) **(Table 6).** These genera showed differing distributions based on type of sample. For example, while all sample types contained Aspergillus spp., only blood specimens yielded Curvularia **(Table 7).**

Frequency of Aspergillus Species

In the genus Aspergillus, A. flavus emerged as the most prevalent species with a dominance of 69.3%, followed by A. niger (14.5%) and A. nidulans (4.8%), with unidentified Aspergillus species making up the remaining 11.2% **(Table 8).** The distribution of A. flavus across sample types showed its highest occurrence in blood (41.9%), followed by urine, sputum, and oral swabs (17.7%, 8.0%, and 1.6%, respectively) **(Table 9).** A. niger and A. nidulans were also predominantly isolated from blood and urine samples.

Morphological Identification

Morphological identification was performed in SDA medium, where colonies were distinct macro- and micro-morphologically conforming to their classification. **(Fig. 1)** shows representative colonies of A. niger, A. flavus, A. nidulans alongside Penicillium, Cladosporium, and Curvularia.

Molecular Identification

For molecular identification, PCR amplification for the aspergillopepsin (PEPO) gene was done on 20 selected Aspergillus isolates. All 20 isolates showed a distinct band near 200 bp, confirming them as A. flavus **(Figure 2).** This was consistent with previous findings using the same primer set.

Analysis of DNA Sequencing and BLAST

A. flavus isolates yielded two PCR products, C1 and C2, which underwent sequencing and comparison with reference sequences in the NCBI database. Sequence alignment with BLAST showed that both C1 and C2 were closely aligned with A. flavus strain AFLA–EME–EG–1, with identities of 98% and 99%, respectively (**Tables 10 and 11**; **Figures 3 and 4**). The results from analytical sequencing were as expected and corroborated the phenotypic and genotypic identification (**Table 12**), which confirms the reliability of molecular diagnostics for Aspergillus spp. identification.

Table (4): Numbers and percentages of samples collected from immune-compromised patients

Type of samples	No. of samples	Percentage (%)
Blood	137	53.5%
Urine	57	22.2%
Oral	32	12.5 %
Sputum	30	11.7 %
Total	256	100%

Table (5): Numbers and percentages of positive growth samples collected from immunocompromised patients.

Type of samples	No. of samples	Percentage (%)
Blood	49	55.6%
Urine	25	28.4%
Oral	4	4.5%
Sputum	10	11.3%
Total	88	100%

Our results revealed five fungal genera isolated from different samples which- included Aspergillus spp. 62(70.4%), Penicillium spp. 18(20.4%), Cladosporium spp. 5(5.6%), Alternaria spp. 2(2.2%) and finally Curvularia spp. 1 (1.1%), Table (6).

Table (6): Frequency percentage and number of fungal genera isolated from immune-compromised patients.

Fungal genera	Percentage of frequency
Aspergillus	70.4%
Penicillium	20.4%
Cladosporium	5.6%
Alternaria	2.2%
Curvularia	1.1%
Total	100%

Also our results revealed the following data which were obtained when collectedsamples from oral, blood, sputum and urine including Aspergillus spp. 36(40.9%), Penicillium spp. 7(7.9%), Cladosporium spp. 4 (4.5%), Alternaria spp. 1(1.1%) and Curvularia spp 1(1.1%) from blood samples while the following genera Aspergillus spp 17(19.3%), Penicillium spp.7 (7.9%), Cladosporium spp. 1(1.1%) obtained from urine samples and also the following isolates Aspergillus spp. 1 (1.1%), Penicillium spp. 2(2.2%) and Alternaria spp. 1 (1.1%) were isolated from oral samples, on the other hand 8 isolates of Aspergillus spp. (9%) and 2 isolates of Penicillium spp. (2.2%) were isolated from sputum, Table (7).

Table (7): Number and percentage of genera isolated from samples distributed according to type of samples.

Samples	Genera	No.	Percentage	Total
Blood	Aspergillus	36	40.9%	49
	Penicillium	7	7.9%	
	Cladosporium	4	4.5%	
	Alternaria	1	1.1%	
	Curvularia	1	1.1%	
Urine	Aspergillus	17	19.3%	25
	Penicillium	7	7.9%	
	Cladosporium	1	1.1%	
Oral	Aspergillus	1	1.1%	4
Sputum	Penicillium	2	2.2%	
	Alternaria	1	1.1%	
Sputum	Aspergillus	8	9%	10
	Penicillium	2	2.2%	
Total			100%	88

The results revealed that Aspergillus molds are more repeated in these samples. Frequency of Aspergillus species were illustrated in Table (8).

Table (8): Frequency percentage and number of Aspergillus spp. isolated fromimmunocompromised patients.

Aspergillus spp.	No. of samples	Percentage of frequency
A.flavus	43	69.3%
A.niger	9	14.5%
A.nidulans	3	4.8%
Other Aspergillus spp.	7	11.2%
Total	62	100%

In addition, A. flavus revealed in the first rank compared with other isolatedspecies in recent study which were 26(41.9%), 11(17.7%), 5(8%) and 1(1.6%) in blood, urine, sputum and oral respectively, while A. niger recorded second rank in percentage 4(6.4%), 3(4.8%) and 2(3.2%) in compared with other Aspergillus spp. In blood, urine and sputum respectively, A. nidulans appeared in blood, urine and sputum and it reached to 1(1.6%), 1(1.6%) and 1(1.6%) respectively, other unidentified Aspergillus appeared in blood and urine reached to 5(8%) and 2(3.2%) respectively, (Table 9).

Table (9): Distribution of Aspergillus spp. isolated independent of samples from immunocompromised patients.

Aspergillus spp.	Samples	NO.	Percentage (%)	Total
A.flavus	Blood Urine Sputum Oral	26 11 5 1	41.9% 17.7% 8% 1.6%	43
A.niger	Blood Urine Sputum	4 3 2	6.4% 4.8% 3.2%	9
A.nidulans	Blood Urine Sputum	1 1 1	1.6% 1.6% 1.6%	3
Other Aspergillus spp.	Blood Urine	5 2	8% 3.2%	7
Total			100%	62

Identification on SDA medium

For fungi cultivated on Sabouraud Dextrose Agar (SDA), the Aspergillus taxonomy is based on macro and micro characteristics. Additionally, a colony's color, texture, exudate and pigment production, as well as its reversal coloration are evaluated in macroscopic examination. Microscopically, identification relies on the conidial head shape and size, degree of serration, vesicle shape and size, stipe length, width, texture, color, and conidia size and form as shown in Figure 1. The conidial heads can be radiate, globose, clavate or columnar (11). Despite the inability to precisely differentiate some species morphologically, it aids in identifying sections or species complexes (12).

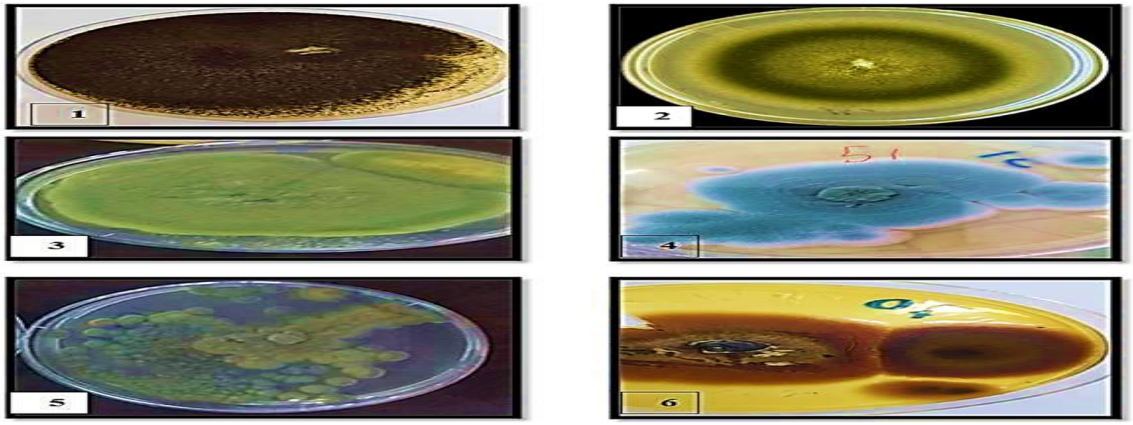

Figure (1): Macrographs showing fungal spp. colonies growing on SDA, 5-7 days 28 Cº, 1:A. niger, 2: Curvularia spp., 3: A. flavus, 4: Penicillium spp., 5: Cladosporium spp. and 6: A. nidulans

Molecular identification

To determine the genotypes 20 Aspergillus spp. isolates the genomic DNAs wereused as templates for PCR using amplified DNA products of aspergillopepsin genewas amplified in case Aspergillus spp.

Genotypic diagnosis of Aspergillus isolates

All 20 Aspergillus spp. isolates were characterized as genotypes using primer PEPO, all isolates were classified as genotype A. flavus. Aspergillopepsin gene amplified product from all A. flavus isolate was (~200 bp) in length, Figure (2).These results were agreement with (5) who found that the PCR product of A.flavus with aspergillopepsin gene was (~200 bp).

Figure (2): Agarose gel electrophoresis of aspergillopepsin gene PCR product by primer PEPO of different Aspergillus spp. Lane (M): DNA ladder; Lane 1-20:A.flavus (1.3g agarose gel, 80 volts for 1 hour)

Sequences analysis

The C1 and C2 strain sequencing data was retrieved electronically and compared with the NCBI database using BLAST software. Sequence editing was done in BioEdit, while the final sequences were submitted to NCBI in FASTA format through Sequin. Both samples' mold DNA was amplified using PEPO primers that target aspergillopepsin gene

Amplified of aspergillopepsin gene by primer PEPO

The C1 strain is found to be the nearest neighbor to A. flavus strain AFLA-EME- EG-1 with identity 98%, Table (3.7) Figure (3.3). While C2 is strain found to be the nearest neighbor to A. flavus strain AFLA-EME-EG-1 with identity 99%, Table (11)and Figure (4). These results were completely identical with our PCR identificationand phenotypic and microscopic diagnosis of molds, Table (12).

Table (10): DNA sequences alignments of isolated C1 (A.flavus) in comparing with database obtained from NCBI website.

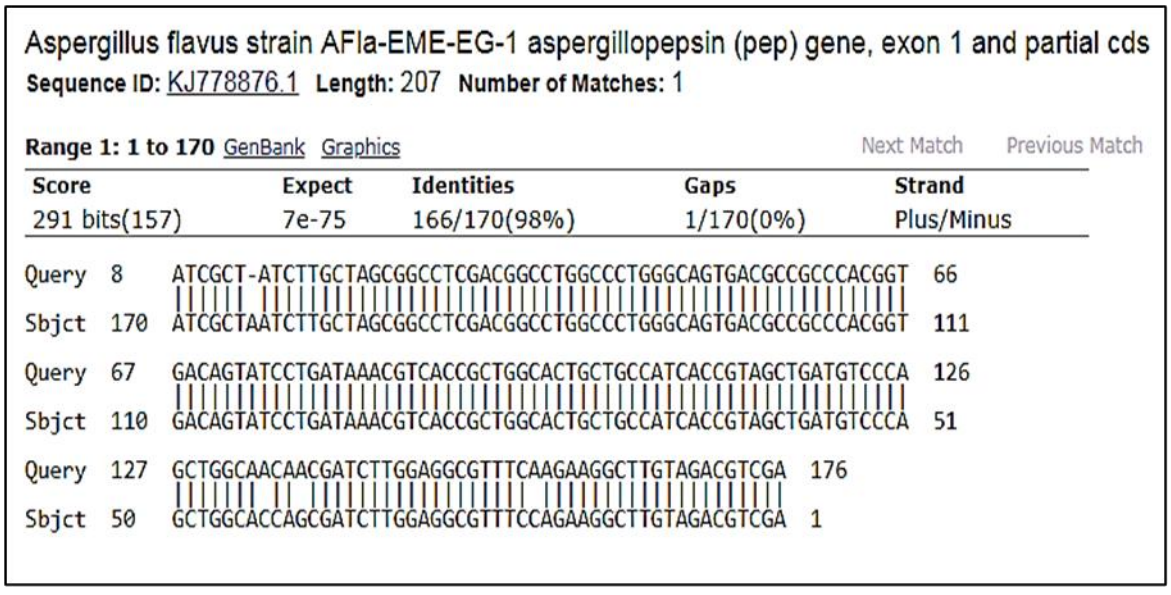

Figure (3): Pairwise alignment of aspergillopepsin gene of C1 (A. flavus) strain AFLA-EME-EG-1 using NCBI online blast.

Table (11): Alignment of DNA sequences of isolated C2 (A. flavus) compared with the database received from the NCBI website.

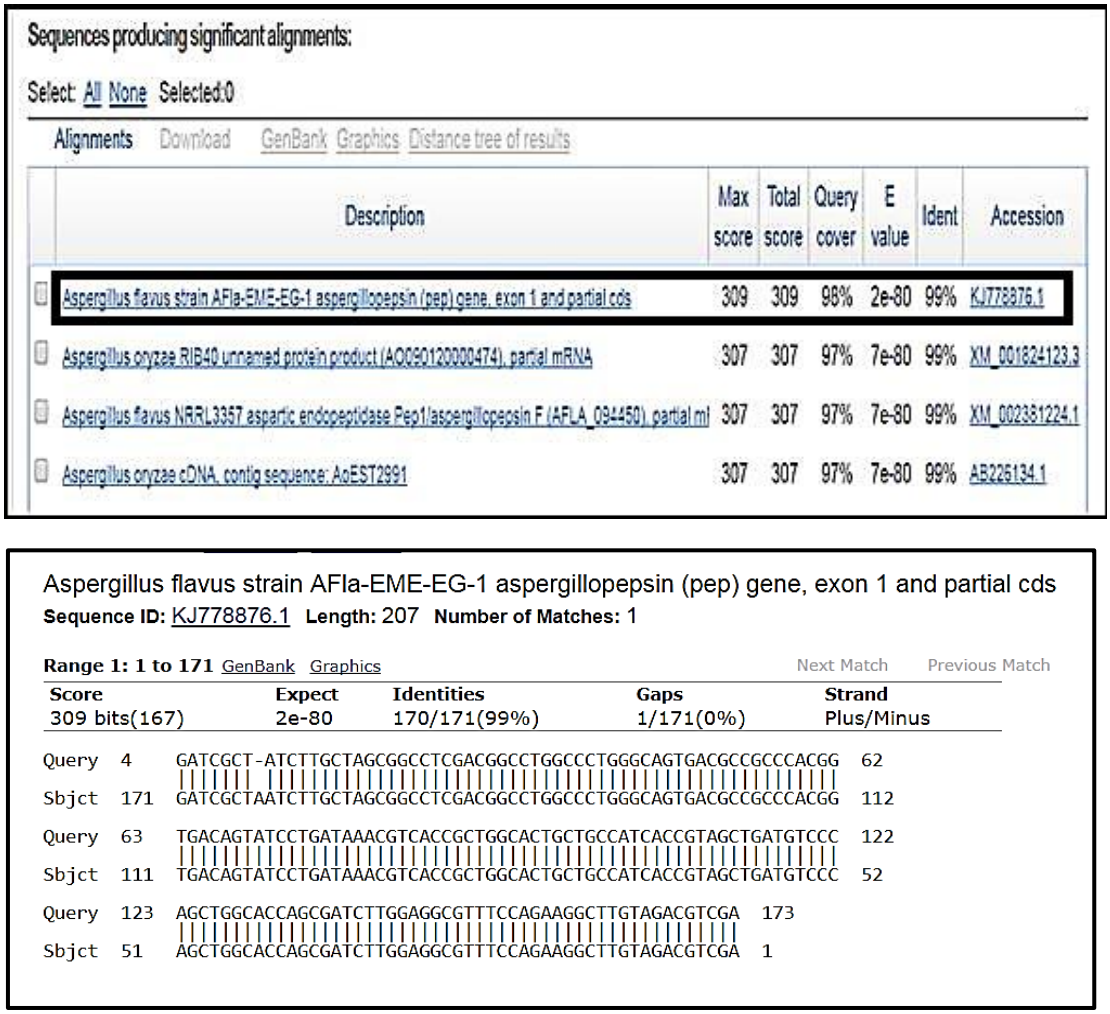

Figure (4): Pairwise alignment of aspergillopepsin gene of C2 (A. flavus) strain AFLA-EME-EG-1 using NCBI online blast

Table (12): Comparison among of phenotypic and genotypic identification with sequence analysis for molds spp.

No.	Phenotypic and Microscopic Diagnosis of molds	Species identification based on PEPO	Species identification (percentage identity with reference sequence PEP sequencing	Final identification
39	A.flavus	A.flavus	A.flavus	A.flavus
40	A.flavus	A.flavus	A.flavus	A.flavus

DISSCUSION

This study illustrates the importance of opportunistic mold infections in immunocompromised hosts, as well as the prevalence of Aspergillus species, mainly A. flavus, among the clinical isolates. From 256 clinical specimens collected, 88 (34.4%) exhibited positive mold growth. Most isolates came from blood samples. These findings corroborate earlier reports identifying blood as a primary site for mold detection in systemic fungal infections, especially in immunocompromised patients (1,2). Aspergillus spp. was among the five fungal genera identified and constituted the majority of the isolates (70.4%), confirming its predominant role in clinical mold infections. This is consistent with the widespread and pathogenic nature of Aspergillus species, particularly in patients with significant immunocompromised (3,4). Within this genus, A. flavus was the most frequent species (69.3%) and was followed by A. niger and A. nidulans. Importantly, A. flavus was isolated from all four types of clinical samples: blood, urine, sputum, and oral swabs, with blood providing the greatest proportion. The predominance of A. flavus may be due to exposure in hot, dry regions, coupled with its ability to produce aflatoxins and proteolytic enzymes, which increases its pathogenicity (5,6).

Morphological identification and classification of molds remains useful as a first step; however, distinguishing characteristics for different species, particularly within groups as morphologically similar as Aspergillus, is often insufficient (10–12). Thus, in this study, integrating molecular diagnostics such as PEPO gene PCR amplification along with sequencing were essential for verifying species identity. All 20 Aspergillus isolates underwent molecular analysis and were confirmed to be A. flavus through PCR, generating ~200 bp amplicons, consistent with prior studies (5). Additional validation through sequencing and comparison to the NCBI database confirmed 98–99% identity with A. flavus strain AFLA-EME-EG-1, bolstering the reliability of the methods used. These findings not only reinforce the reliability of the integrated phenotypic and molecular approach for mold identification but also underscore the pressing need for establishing a regional reference database. This need is particularly acute for Iraq, where information about fungal pathogens in clinical contexts is limited. Creating such localized databases improves identification precision, supports antimicrobial stewardship by decreasing misidentification, and enhances epidemiological surveillance.

CONCLUSION

This investigation offers strong confirmation regarding the prevalence of Aspergillus flavus within mycotic infections of the immunocompromised patients in an Iraqi clinical setting. Phenotypic characterization corroborated by PCR and DNA sequencing substantiated the presence of A. flavus along with its genetic identity from other reference strains around the world. The incorporation of molecular diagnosis not only improved the precision of the identification of the fungi but also made it possible to construct a local registry of clinically important fungi. These findings reinforce the need to regionally monitor the infection data to improve diagnostic procedures and underscore the necessity for the use of molecular tools in the infection control and clinical management of the region. Further work

with greater sample size, antifungal susceptibility, and deeper genomic profiling is needed to clarify the pathogenicity and antifungal resistance of these molds in susceptible populations.

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Ethical Considerations

This study was conducted according to the principles of the Declaration of Helsinki which stipulates ethical guidelines for physiological and medical research. All subjects were provided with clear participant information sheets and gave their written informed consent prior to participation.

Conflicts of Interest

No competing interests have been declared by the authors for this manuscript from other sources, financial or otherwise.

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